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VISTA Deficiency Accelerates the Development of Fatal Murine Lupus Nephritis

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Abstract

Objective—The targeting of negative checkpoint regulators as a means of augmenting antitumor immune responses is now an increasingly used and remarkably effective approach to the treatment of several human malignancies. The negative checkpoint regulator VISTA (V-domain Ig-containing suppressor of T cell activation; also known as programmed death 1 homolog or as death domain 1 α) suppresses T cell responses and regulates myeloid activities. We proposed that exploitation of the VISTA pathway is a novel strategy for the treatment of human autoimmune disease, and therefore we undertook this study to determine the impact of VISTA genetic deficiency on lupus development in a lupus-prone mouse strain.

Methods—To evaluate whether genetic deficiency of VISTA affects the development of lupus, we interbred VISTA-deficient mice with *Sle1.Sle3* mice, a well-characterized model of systemic lupus erythematosus (SLE).

Results—We demonstrated that the development of proteinuria and glomerulonephritis in these mice, designated *Sle1.Sle3* VISTA^{−/−} mice, was greatly accelerated and more severe compared to that in *Sle1.Sle3* and C57BL/6 VISTA^{−/−} mice. Analysis of cells from *Sle1.Sle3* VISTA^{−/−} mice showed enhanced activation of splenic CD4⁺ T cells and myeloid cell populations. No increase in titers of autoantibodies was seen in *Sle1.Sle3* VISTA^{−/−} mice. Most striking was a significant increase in proinflammatory cytokines, chemokines, and interferon (IFN)–regulated genes associated with SLE, such as IFN α , IFN γ , tumor necrosis factor, interleukin-10, and CXCL10, in *Sle1.Sle3* VISTA^{−/−} mice.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Ceeraz and Noelle had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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ADDITIONAL DISCLOSURES

Authors Pechenick and Noelle are employees of ImmuNext, Inc.

Conclusion—This study demonstrates for the first time that loss of VISTA in murine SLE exacerbates disease due to enhanced myeloid and T cell activation and cytokine production, including a robust IFN α signature, and supports a strategy of enhancement of the immunosuppressive activity of VISTA for the treatment of human lupus.

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease (1–3). SLE is characterized by a host of immunologic abnormalities, notably loss of tolerance to nuclear components and defective clearance of apoptotic cells resulting in exposure of autoantigens (4). The pathogenesis of lupus nephritis includes the production of anti–double-stranded DNA (anti-dsDNA) antibodies, immune complex (IC) deposition in the kidneys, and progressive glomerular damage (5). Treatment of lupus nephritis is generally effective but toxic, and there remains a large unmet need for potent but safe therapeutics in this disease (6).

Targeting of negative checkpoint regulators, including programmed death 1 (PD-1) and CTLA-4, as a means of augmenting antitumor immune responses in vivo is now an increasingly used and remarkably effective approach to the treatment of several malignancies in humans (7,8). In our laboratory and those of other investigators, the negative checkpoint regulator VISTA (V-domain Ig–containing suppressor of T cell activation [9,10]; also known as PD-1 homolog [11,12] or as death domain 1 α [DD-1 α] [13]) has been extensively characterized (9,10,12,14). VISTA is expressed on CD11b^{high} myeloid cells, neutrophils, natural killer cells, and T cells (9). VISTA shares homology with PD ligand 1 and PD-1 (9,12) and can suppress T cell activation in vitro (9). Recently, VISTA was shown to affect the activities of myeloid cells as a receptor (15), and a very recent study showed involvement of VISTA in the uptake of apoptotic cells (13). These data suggest that VISTA activities could exert a profound effect on the development and progression of lupus.

We and others have reported a mild proinflammatory phenotype in VISTA^{−/−} mice on the nonautoimmune C57BL/6 (B6) background (11,16,17), with an increase in T cell activation markers and cytokine production. A significant increase in CD11b+MHCII+ cells and myeloid dendritic cells (DCs) is seen, indicative of enhanced myeloid cell activities in the absence of VISTA (17). In contrast, offspring of VISTA^{−/−} mice interbred with the experimental autoimmune encephalomyelitis (EAE)–susceptible strain 2D2 T cell receptor–transgenic mice (2D2 mice), where the T cell receptor is specific for myelin oligodendrocyte glycoprotein 35–55 peptide, develop severe EAE (17). Experiments involving transfer of VISTA^{−/−} 2D2 mouse cells into recombination-activating gene 1 (RAG-1)–deficient VISTA^{−/−} recipient mice suggest that the absence of VISTA on recipient mouse myeloid cells exacerbates the development of autoimmunity. However, in EAE, it appears that the absence of VISTA on the encephalitogenic T cells also contributes to the enhancement of disease (17). The role of VISTA in controlling autoimmunity is also apparent in a hepatitis model in which enhanced inflammation and expanded T cell responses are seen in the absence of VISTA (11). In this latter model, VISTA expression on T cells appears to play a key role in modulating disease.

Because VISTA affects the development of autoimmunity, specifically by regulating myeloid and T cell activities, studies were designed to evaluate the influence of VISTA in a

murine model of lupus. *Sle1.Sle2.Sle3* congenic mice have 3 susceptibility loci from NZM2410 lupus-prone mice, which were introgressed into nonautoimmune B6 mice. While tricongenic *Sle1.Sle2.Sle3* mice spontaneously develop aggressive disease, bicongenic *Sle1.Sle3* mice have reduced penetrance and severity of lupus nephritis (18). We show for the first time that *Sle1.Sle3* mice with the genetic absence of VISTA (*Sle1.Sle3* VISTA^{-/-} mice) develop a profoundly accelerated and fatal form of lupus nephritis as compared with VISTA-intact *Sle1.Sle3* mice. Extensive analysis revealed that VISTA deficiency primarily affects the myeloid and T cell compartment, imparting an up-regulation of costimulatory and activation markers and heightened proinflammatory cytokine production compared to control *Sle1.Sle3* mice. These results support a strategy of exploiting the VISTA pathway for treatment of human autoimmune disease, including lupus.

MATERIALS AND METHODS

Mice

B6.NZM*Sle1.Sle2.Sle3*, (NZB × NZW)F1, and NZM2410 mice were purchased from The Jackson Laboratory, and female B6 mice were purchased from the National Cancer Institute (Frederick, MD). B6.VISTA^{-/-} mice were bred as described previously (17). To generate *Sle1.Sle3* VISTA^{-/-} mice, male and female B6.NZM*Sle1.Sle2.Sle3* and VISTA^{-/-} mice were interbred. According to instructions from the laboratory of Dr. Laurence Morel (Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville), genotyping was performed to identify *Sle1* (D1Mit47, D1Mit15, D1Mit113, and D1Mit155), *Sle2* (D4Mit6, D4Mit329, and D4Mit72), and *Sle3* (CKMM, D7Mit157, and D7Mit40) loci. The absence of all markers for *Sle2* was confirmed by genotyping. This was further confirmed by a loss of the “tan” coat color appearance. *Sle1.Sle3* VISTA^{-/-} mice were all gray. All mice were housed in the pathogen-free facility at the Geisel School of Medicine at Dartmouth.

Proteinuria

Levels of proteinuria (in mg/dl) were recorded weekly using Chemstrip test strips (Roche Diagnostics) beginning when the mice were 6 weeks old. Body weight was also recorded at that time.

Antibodies

All antibodies for flow cytometry were purchased from BioLegend. These included antibodies against B220, CD3, CD4, CD8, CD11b, CD11c, CD16, CD19, CD40L, CD80, CD25, CD40, CD44, CD62L, CD86, CD69, CXCR5, I-A^b, IgG, GL7, PD-1, inducible costimulator, CTLA-4, NK1.1, Gr-1, F4/80, and CD45. To detect FoxP3⁺ Treg cells, we used a mouse Treg cell staining kit (eBioscience). We stained for expression of VISTA using an allophycocyanin-conjugated antibody (13F3) from our laboratory (9). For immunofluorescence staining, we used antibodies to detect IgG (Invitrogen), C3 (MP Biomedicals), F4/80, and CD11b.

Toll-like receptors (TLRs) and recombinant proteins

For in vitro stimulation assays, agonists for TLR-7 (R848; Invitrogen) and TLR-9 (CpG; Invitrogen) were used at 1 µg/ml. Lipopolysaccharide (LPS) at 10 ng/ml (Sigma-Aldrich) and interferon- α (IFN α) at 1,000 units/ml (Hycult Biotechnology) were also used.

Kidney pathology

For clinical pathology, kidneys were fixed in formalin for paraffin embedding. Sections (4 mm) were stained with hematoxylin and eosin and examined in a blinded manner by one of us (ARS).

Cytokine and chemokine analysis

Serum was collected from retroorbital eye bleeds and screened by Luminex assay. The assay was performed using a 32 Milliplex Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore), run on a Bio-Plex 200 System (Bio-Rad), and quantified using Bio-Plex Manager 6.0 software (Bio-Rad). Enzyme-linked immunosorbent assay (ELISA) kits were purchased to screen for antidsDNA antibodies (BioVendor Research and Diagnostic Products), anti-dsDNA IgG (Alpha Diagnostic), and antihistone Ig (Alpha Diagnostic).

Flow cytometric analysis

Flow cytometry was performed as previously described (9,19). Cells were acquired on a MACSQuant Analyzer 10 (Miltenyi Biotec) and analyzed using FlowJo software (Tree Star).

Kidney cell isolation

Kidney cells were obtained using a Miltenyi Biotec gentleMACS Dissociator protocol.

Immunofluorescence staining

OCT compound-embedded frozen kidneys were sectioned and stored at -80°C until processing. For staining, slides were fixed in prechilled acetone for 10 minutes in Coplin jars. Excess acetone was removed, and slides were rehydrated in phosphate buffered saline (PBS). Slides were placed in a humid box and incubated with 10% goat serum (Jackson ImmunoResearch) in PBS for 1 hour at room temperature, washed in PBS, stained with antibodies for 2 hours at room temperature, and mounted with ProLong Gold Antifade Mountant with DAPI (Life Technologies). Images were acquired on a Zeiss LSM 510 Meta Confocal Microscope and analyzed with LSM 510 Meta software.

Cell sorting

Spleens were harvested and stained with the appropriate antibodies as previously described. Myeloid cells and T cells were sorted on a BD FACSAria III (BD Biosciences).

Bone marrow-derived DCs (BMDCs) and macrophage generation

BMDCs and BM macrophages were generated as previously described (20). Macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and interleukin-4 (IL-4) were used (all at 10 ng/ml; all from R&D Systems).

Heatmaps

Heatmaps were generated from cytokine concentrations using the heatmap.2 function in the gplots package (Various R programming tools for plotting data; R package version 2.15.0) of the R statistical programming language (R Foundation for Statistical Computing; <https://www.R-project.org>). Agglomerative hierarchical clustering (unweighted pair group method with arithmetic mean) was used to build the cytokine dendrogram, with the distance between a pair of cytokines defined as $1 - r$, where r is the Pearson coefficient of correlation between the concentrations of the 2 cytokines. Row scaling was then performed for each cytokine, and Z scores were plotted as a heatmap.

Statistical analysis

Graphs were generated with GraphPad Prism software version 6. Statistical analysis was performed using Student's unpaired t -test.

RESULTS

Reduced VISTA expression during active disease in *Sle1.Sle3* mice

Previous studies have shown that VISTA is highly expressed in the myeloid cell compartment and at lower densities on resting T cells (9,10). In the present study, we examined VISTA expression by flow cytometry (see Supplementary Figures 1A–E, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.40020/abstract>) on cell populations in *Sle1.Sle3* mice during disease progression, starting with 8-week-old premorbid mice without proteinuria and ending with 40-week-old sick mice with proteinuria (100–500 mg/dl). Age- and sex-matched B6 mice were used as controls.

The percentage of inflammatory monocytes (F4/80+Gr-1+CD11b^{high}) that expressed VISTA did not differ between young and old mice. However, in *Sle1.Sle3* mice with advanced disease, the percentage of inflammatory monocytes that expressed VISTA was significantly reduced (Figure 1A). In contrast to its expression on myeloid cells, VISTA expression on activated T cells declined significantly with age both in control B6 mice and in *Sle1.Sle3* mice (Figure 1B). In terms of absolute numbers, a significant decline in VISTA expression on splenic inflammatory monocytes and activated T cells was seen only in older, sick *Sle1.Sle3* mice (see Supplementary Figures 1F and G, <http://onlinelibrary.wiley.com/doi/10.1002/art.40020/abstract>). As anticipated (9,10), no VISTA expression on B cells was apparent (results not shown). In summary, we identified a disease-specific reduction in VISTA expression in the inflammatory monocyte compartment of lupus-prone mice as compared to B6 controls.

VISTA deficiency exacerbates proteinuria and glomerulonephritis in lupus-prone *Sle1.Sle3* mice

The finding that VISTA expression on inflammatory monocytes declines as lupus progresses in *Sle1.Sle3* mice led us to postulate that a genetically induced loss of VISTA expression might greatly accelerate and/or exacerbate disease. To address this, VISTA^{-/-} mice were interbred with *Sle1.Sle3* mice, and disease progression was assessed by proteinuria in age-matched B6, B6 VISTA^{-/-}, *Sle1.Sle3*, and *Sle1.Sle3* VISTA^{-/-} mice. Proteinuria levels were

significantly higher in *Sle1.Sle3* VISTA^{-/-} mice compared with *Sle1.Sle3* or B6 VISTA^{-/-} mice (e.g., at 20 weeks, mean $\pm$ SEM 366.7 $\pm$ 84.3 mg/dl in *Sle1.Sle3* VISTA^{-/-} mice versus 48.5 $\pm$ 16.9 mg/dl in *Sle1.Sle3* mice; $P = 0.04$ [n = 6 mice per group]) (Figure 1C). No proteinuria was detected in B6 or B6 VISTA^{-/-} mice at any time point. *Sle1.Sle3* VISTA^{-/-} mice had dramatically reduced survival compared to *Sle1.Sle3* and control mice (Figure 1D), with all *Sle1.Sle3* VISTA^{-/-} mice dead by 25 weeks. One hundred percent of mice in all other groups, including *Sle1.Sle3* mice, were alive at 48 weeks.

To determine the impact of VISTA deficiency on renal pathology, kidney sections were semiquantitatively graded for inflammation. A significant increase in the interstitial infiltrate score was found in *Sle1.Sle3* VISTA^{-/-} mice compared to *Sle1.Sle3* mice (mean $\pm$ SEM 3 $\pm$ 0% versus 1.17 $\pm$ 0.4%; $P = 0.02$) (see Supplementary Figure 2A, <http://onlinelibrary.wiley.com/doi/10.1002/art.40020/abstract>), but no difference in glomerular score was detected (3 $\pm$ 0% versus 1.67 $\pm$ 0.4%, respectively). The fibrosis score was also significantly increased in *Sle1.Sle3* VISTA^{-/-} mice (0.83 $\pm$ 0.2% versus 0.17 $\pm$ 0.2%; $P = 0.04$). In general, the clinical pathology of kidney sections consistently showed severe glomerulonephritis in *Sle1.Sle3* VISTA^{-/-} mice compared with B6 VISTA^{-/-} mice or *Sle1.Sle3* mice (Supplementary Figure 2B). These findings demonstrate that in the absence of immunoregulatory VISTA in *Sle1.Sle3* mice, the progression and severity of lupus nephritis are greatly enhanced.

To determine whether disease exacerbation observed in *Sle1.Sle3* VISTA^{-/-} mice was related to increased production of autoreactive antibodies or to their nature, titers of specific serum antibodies to dsDNA (total) (Supplementary Figure 3A), IgG antidsDNA (Supplementary Figure 3B), and histones (total) (Supplementary Figure 3C) were quantified. We detected no difference between the *Sle1.Sle3* mice and the *Sle1.Sle3* VISTA^{-/-} mice. No differences were found in the frequencies of splenic activated B cells (Supplementary Figure 3D), germinal center B cells (Supplementary Figure 3E), or IgG-producing B cells (Supplementary Figure 3F) between *Sle1.Sle3* VISTA^{-/-} mice and B6 VISTA^{-/-} or *Sle1.Sle3* mice. In parallel, IC (C3/IgG) deposition was examined in kidneys, and no difference was found between kidneys from *Sle1.Sle3* mice and those from *Sle1.Sle3* VISTA^{-/-} mice (Supplementary Figure 2C).

VISTA deficiency on the *Sle1.Sle3* background elevates proinflammatory cytokines in the serum

To evaluate the systemic effects of VISTA deficiency within the context of the *Sle1.Sle3* genotype, serum levels of cytokines and chemokines were quantified in B6, B6 VISTA^{-/-}, *Sle1.Sle3*, and *Sle1.Sle3* VISTA^{-/-} mice. A significant increase in mediators associated with disease pathogenesis, including IL-10, tumor necrosis factor (TNF), IFN γ , and IL-1 α , was found in *Sle1.Sle3* VISTA^{-/-} mice compared to B6, B6 VISTA^{-/-}, or *Sle1.Sle3* mice (Figure 1E). A mild proinflammatory phenotype was also noted in B6 VISTA^{-/-} mice compared to B6 mice, as previously described (17). Thus, VISTA deficiency enhances systemic inflammation in *Sle1.Sle3* mice.

Increased frequencies of activated T cells in *Sle1.Sle3* VISTA^{-/-} mice compared to *Sle1.Sle3* mice

Previous studies have shown that VISTA can negatively regulate T cell proliferation and activation (9) and that T cells from VISTA^{-/-} mice express heightened levels of activation markers (17). Splenic phenotypic analysis in our study revealed modest increases in 25-week-old *Sle1.Sle3* VISTA^{-/-} mice compared with 25-week-old *Sle1.Sle3* mice, both in effector CD4⁺ T cells ($59.3 \pm 3.4\%$ versus $49.2 \pm 2.3\%$; $P = 0.03$) (Figure 2A) and in activated CD4⁺ T cells expressing CD69 ($75.5 \pm 4.5\%$ versus $61.6 \pm 3.6\%$; $P = 0.04$) (Figure 2B). No difference in CD4⁺ Treg cells was found (Figure 2C), but a modest increase in follicular helper T cells was found in *Sle1.Sle3* VISTA^{-/-} mice (Figure 2D).

Th1 and Th2 immune responses are aberrant in SLE (21). To determine whether VISTA deficiency affected T helper cell differentiation, ex vivo splenic CD3⁺CD4⁺ T cells were activated with CD3 and CD28 and screened for Th1 and Th2 cytokines. At 24 hours, IFN γ , IL-4, IL-6, IL-10, and IL-13 were significantly elevated in *Sle1.Sle3* VISTA^{-/-} mice (Figure 2E). No differences in levels of IL-2 or IL-17 were found. To determine whether this was dependent on cell differentiation, transcription factors for Th1 and Th2 differentiation were assessed, and no differences were detected (data not shown).

VISTA deficiency on the *Sle1.Sle3* background up-regulates costimulatory/activation molecules and proinflammatory cytokines in the splenic myeloid compartment

We next examined changes in the splenic myeloid compartment in *Sle1.Sle3* VISTA^{-/-} mice. In parallel with changes in the T cell compartment, the percentage of splenic inflammatory monocytes was significantly higher in *Sle1.Sle3* VISTA^{-/-} mice (Figure 3A). To assess myeloid activation markers in VISTA deficiency, expression of CD80, CD40, and I-A^b was examined in splenic lymphoid DCs (CD11c^{high}CD8^{high}CD11b^{intermediate}) (Figure 3B), myeloid DCs (CD11c^{high}CD11b^{high}CD8^{low} Gr-1^{intermediate}) (Figure 3C), and macrophages (F4/80^{high}CD11b^{intermediate}CD11c^{intermediate}) (Figure 3D). In all 3 groups, expression was significantly greater in *Sle1.Sle3* VISTA^{-/-} mice compared with B6 VISTA^{-/-} or *Sle1.Sle3* mice. This heightened activation status correlated functionally with increases in IL-10 production (Figure 3E) and IFN γ production (Figure 3F), but not TNF production (Figure 3G), by purified ex vivo splenic inflammatory monocytes in response to in vitro stimulation with LPS. These increases in IL-10 and IFN γ mimicked those seen previously in the serum of *Sle1.Sle3* VISTA^{-/-} mice (Figure 1E).

VISTA deficiency on the *Sle1.Sle3* background enhances myeloid infiltration into nephritic kidneys

Infiltration of myeloid cell populations displaying a proinflammatory phenotype into the kidneys of mice with active nephritis has been well characterized (22). To assess whether VISTA deficiency altered the infiltration of myeloid cells into the kidneys, renal infiltrating cells were analyzed by flow cytometry as well as by immunofluorescence staining of frozen kidney specimens. Immunofluorescence staining revealed an abundance of CD11b⁺ and F4/80⁺ cells in *Sle1.Sle3* VISTA^{-/-} mice compared with *Sle1.Sle3* mice (Figure 4A). In parallel, flow cytometric analysis showed a significant increase in Gr-1⁺ CD11b⁺ cells (Figure 4B).

Heightened proinflammatory phenotype in *Sle1.Sle3* VISTA^{-/-} mouse BMDCs

A mild inflammatory phenotype in VISTA^{-/-} mice has been reported (17). To determine whether VISTA plays an intrinsic role in myeloid cells in addition to T cells, BMDCs and BM macrophages were generated ex vivo from BM precursors and assessed for the expression of activation markers in response to LPS stimulation. Expression of CD80, but not CD16 or I-A^b, on BMDCs was significantly increased in *Sle1.Sle3* VISTA^{-/-} mice compared with *Sle1.Sle3* mice (Figure 5A). Expression of CD80 and CD40 was also increased on BM macrophages (Figure 5B). To assess whether an increased activation phenotype was associated with augmented effector function, supernatants from LPS-activated BMDC and BM macrophage cultures were screened. Levels of IL-12 (Figure 5C) and TNF (Figure 5D) were modestly yet significantly increased in BMDCs from *Sle1.Sle3* VISTA^{-/-} mice compared to those from *Sle1.Sle3* mice. There was no significant increase in IL-12 production by BM macrophages (Figure 5E); however, as with BMDCs, there was a significant increase in TNF production by LPS-activated BM macrophages from *Sle1.Sle3* VISTA^{-/-} mice compared to those from *Sle1.Sle3* mice (Figure 5F).

More robust IFN signature in *Sle1.Sle3* VISTA^{-/-} mice

In several murine models of lupus, including MRL/lpr, (NZB × NZW)F1, and *Sle1.Sle2.Sle3* mice, high levels of IFN α - and IFN γ -responsive genes are expressed (23–25). We examined whether VISTA deficiency exacerbates this so-called “IFN signature” in *Sle1.Sle3* mice. Indeed, by ELISA, IFN α levels were elevated in the serum of *Sle1.Sle3* VISTA^{-/-} mice compared to that of *Sle1.Sle3* or B6 VISTA^{-/-} mice (Figure 6A). We next stimulated BMDCs in vitro with IFN α (Figure 6B), with R848 (which binds TLR-7) (Figure 6C), or with CpG (which binds TLR-9) (Figure 6D), and we measured the levels of CXCL10 and IFN β (both of which are classic IFN-responsive gene products) in supernatants at 24 hours by Luminex assay. In lupus, ICs containing nuclear components are recognized by TLR-7 and TLR-9, which in part drives disease progression (26,27). In support of the notion of VISTA-mediated regulation of IFN responsiveness in this model of lupus, levels of CXCL10 and IFN β were modestly elevated in *Sle1.Sle3* VISTA^{-/-} mice compared to *Sle1.Sle3* or B6 VISTA^{-/-} mice in response to stimulation with IFN α (Figure 6B), R848 (TLR-7) (Figure 6C), or CpG (TLR-9) (Figure 6D).

DISCUSSION

The data presented herein are the first to demonstrate the role of VISTA in regulating the development of murine SLE. In this study, we first showed that VISTA expression on activated CD4⁺ T cells is generally reduced with age, but that is not the case with inflammatory monocytes. However, VISTA expression on inflammatory monocytes was stable with age in B6 mice, but significantly reduced in nephritic mice with disease progression (Figure 1). Furthermore, a significant decline in the absolute number of splenic inflammatory monocytes and activated T cells with normal VISTA expression was seen only in older, sick *Sle1.Sle3* mice and not in B6 controls (see Supplementary Figures 1F and G, <http://onlinelibrary.wiley.com/doi/10.1002/art.40020/abstract>). At present, the factors and environmental triggers at the transcriptional and posttranscriptional levels involved in regulating VISTA expression are unclear. One study has shown that DD-1 α (VISTA) is a

downstream postapoptotic target of the transcription factor p53 and that expression is enhanced by genotoxic stress (13). Furthermore, we have observed heightened VISTA expression in the tumor microenvironment and in response to retinoic acid and hypoxia (Mabaera R: unpublished observations).

We then demonstrated that *Sle1.Sle3* mice bred onto a VISTA-deficient background experience significantly accelerated fatal glomerulonephritis, with pronounced enhancement of the systemic autoimmune phenotype imparted by the *Sle1* and *Sle3* loci. Morel and Wakeland demonstrated that the combination of *Sle1* and *Sle3* loci results in development of systemic autoimmunity and variably penetrant, severe glomerulonephritis (28). The mortality rate in the original bicongenic *Sle1.Sle3* cohort was 25% at 1 year, with 50% of all mice demonstrating proliferative glomerulonephritis on renal pathology at termination (28). In our survival cohort, the lack of VISTA dramatically worsened outcome, with all *Sle1.Sle3* VISTA^{-/-} mice dead by week 25, while 100% of *Sle1.Sle3* mice were still alive at 48 weeks (Figure 2B). Highly significant increases in TNF, IL-1 α , IL-10, and IFN γ were apparent in the serum of *Sle1.Sle3* VISTA^{-/-} mice compared to all other groups, suggesting a highly inflammatory systemic environment.

Investigators in our laboratory had previously shown that offspring of VISTA^{-/-} mice interbred with 2D2 mice develop severe EAE (17). Similar to the findings in the VISTA^{-/-} 2D2 mice, we observed increased IFN γ production by CD4⁺ T cells in *Sle1.Sle3* VISTA^{-/-} mice. In addition, production of IL-10 and TNF by ex vivo-stimulated CD4⁺ T cells harvested from *Sle1.Sle3* VISTA^{-/-} mice was also increased, the former significantly so (Figure 2E), and this may be the basis for the elevated levels of these cytokines in the serum of sick mice. Taken together, these data suggest that VISTA plays an important role in regulating autoimmunity, particularly the production of a fairly restricted number of pathogenic cytokines: IFN γ in mice with EAE and *Sle1.Sle3* mice and IL-10 in *Sle1.Sle3* mice. In EAE, VISTA expression is detected on both myeloid and T cells, and VISTA-deficient 2D2 mice develop severe EAE (17). Analysis of cells from the central nervous system showed an increase in IL-17A⁺ and IFN γ ⁺ T cells in VISTA^{-/-} 2D2 mice. Furthermore, experiments involving transfer of VISTA^{-/-} 2D2 mouse cells into RAG-1-deficient VISTA^{-/-} recipient mice suggested that the absence of VISTA on recipient mouse myeloid cells exacerbates the development of autoimmunity. Collectively, these data demonstrated a crucial role of VISTA in the myeloid and T cell compartments (17).

An increased percentage of inflammatory monocytes was found in the spleens of 25-week-old *Sle1.Sle3* VISTA^{-/-} mice compared with other groups (Figure 3A). In addition, elevated expression of multiple costimulatory/activation molecules was seen (Figures 3B–D). Furthermore, ex vivo elaboration of several proinflammatory cytokines from in vitro-stimulated inflammatory myeloid cells was found, with significant increases in IL-10 and IFN γ (Figures 3E and F) as well as a statistically nonsignificant increase in TNF (Figure 3G). This heightened inflammatory phenotype may contribute to the same elevated cytokine levels that were observed in the serum of nephritic *Sle1.Sle3* VISTA^{-/-} mice. In addition, we documented increased myeloid cell infiltration into kidneys of *Sle1.Sle3* VISTA^{-/-} mice (Figure 4) and enhanced myeloid activation in the BM (Figure 5). Cumulatively, these

findings suggest that an activated myeloid compartment in the absence of VISTA contributes to the intensified disease seen in *Sle1.Sle3* VISTA^{-/-} mice.

Type I IFNs have been linked with SLE pathogenesis, the so-called “IFN signature” (29,30). Lupus patients have high levels of IFN α in their serum (31,32) and increased expression of IFN-responsive genes (29). The IFN signature has been reported in *Sle1.Sle2.Sle3* mice (23). We found that IFN α levels were significantly increased in *Sle1.Sle3* VISTA^{-/-} mice compared with *Sle1.Sle3* or B6 VISTA^{-/-} mice (Figure 6A). Furthermore, in vitro stimulation of *Sle1.Sle3* VISTA^{-/-} mouse BMDCs with recombinant IFN α resulted in elevation of IFN β and CXCL10 (Figure 6B), the latter of which correlates positively with the presence of lupus nephritis (33). We also found that CXCL10 production by *Sle1.Sle3* VISTA^{-/-} mouse BMDCs was increased by stimulation with TLR-7 (Figure 6C) and TLR-9 (Figure 6D). These findings are similar to the high levels of CXCL10 reported in TLR-7- and TLR-9-stimulated *Sle1.Sle2.Sle3* mouse BMDCs (23), suggesting that VISTA may regulate myeloid activation in the BM.

A previous study has shown that IFN α interferes with the therapeutic action of CTLA-4Ig in (NZB $\times$ NZW)F1 mice (34). At present, we do not know the precise mechanisms by which VISTA deficiency contributes to heightened type I IFN in *Sle1.Sle3* VISTA^{-/-} mice. We are currently investigating which cell populations are producing these mediators as well as their implications for lupus progression. VISTA does not appear to be mediating its regulatory effects directly on the B cell compartment (see Supplementary Figure 2C, <http://onlinelibrary.wiley.com/doi/10.1002/art.40020/abstract>). Clearly, the most striking effects are on the myeloid and T cell compartments.

In our laboratory, we have shown that an anti- VISTA monoclonal antibody, 13F3, facilitates antitumor immunity and exacerbates disease in EAE (9). Recent data have suggested that in addition to its role as a ligand on myeloid cells, VISTA can also act as a receptor on myeloid cells. For example, spontaneous cytokine production was found in monocytes from HIV-infected individuals that overexpressed VISTA. Furthermore, these monocytes were able to increase cytokine production by HIV-specific T cells (15). Investigators in our laboratory have shown that VISTA functions as a ligand on myeloid cells to suppress T cell activation through a putative VISTA receptor (9,10). Others have shown that VISTA functions as a receptor, as demonstrated by the binding of an anti-VISTA antibody to VISTA+ T cells to mediate T cell suppression (11,12).

VISTA^{-/-} mice on the B6 background do not develop SLE, as reported by 3 independent laboratories, including our own (11,17,35). In our studies of VISTA^{-/-} mice, 3 organs (lung, liver, and pancreas) were found to contain significant numbers of immune cell infiltrates. However, despite the presence of chronic inflammation in multiple tissues, aged (~1-year-old) VISTA^{-/-} mice did not develop overt organ-specific autoimmune disease. This is further supported by no significant increase in serum autoantibodies and no IC deposition in the glomeruli. These findings indicate that VISTA deficiency does not result in any obvious autoimmunity in the absence of other predisposing factors. In contrast, however, a recent study has shown that 10-month-old DD-1 α (VISTA)-deficient B6 mice develop spontaneous glomerulonephritis (13). At this time, it is not clear why there is a difference in

disease penetrance across these VISTA^{-/-} mice. However, this could be due to environmental factors, including animal housing, the method used for generating the deficient mice (one conditional and one gene deleted), or other as-yet-unknown reasons. Finally, the enhanced disease conferred by VISTA deficiency in *Sle1.Sle3* mice despite the continued presence of other immunoregulatory molecules is similar to the exacerbated disease severity in VISTA^{-/-} 2D2 mice (17).

In conclusion, our study demonstrates for the first time that the VISTA pathway plays a critical role in modulating the innate and adaptive immune response in the *Sle1.Sle3* murine model of lupus. VISTA deficiency in this model results in an enhanced myeloid and T cell inflammatory phenotype, a more robust type I IFN signature, accelerated glomerulonephritis, and early mortality. This occurs despite the ongoing presence of other negative checkpoint regulators, which are unable to compensate for the loss of VISTA. The data presented herein establish a unique role of VISTA in regulating autoimmunity, and they support manipulation of the VISTA pathway to treat human autoimmune disease, particularly lupus. We are currently interbreeding *Sle1.Sle3* mice with VISTA-conditional knockout mice. Once they become available, these mice will allow for a more specific assessment of the relative contributions of VISTA regulation of the myeloid or T cell compartments in preventing disease progression in this model. Studies are also underway to identify the counter-receptor for VISTA, which will greatly enhance our ability to dissect the role of the VISTA pathway in lupus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Expression of VISTA (V-domain Ig-containing suppressor of T cell activation) is reduced during active lupus, and VISTA deficiency exacerbates proteinuria and glomerulonephritis in lupus-prone *Sle1.Sle3* (*Sle1, 3*) mice. Spleens were harvested from female *Sle1.Sle3* mice without proteinuria, sick female *Sle1.Sle3* mice with proteinuria (500 mg/dl), and female C57BL/6 (B6) control mice. **A** and **B**, Cells were stained with directly conjugated antibodies to identify VISTA expression on inflammatory monocytes (F4/80+Gr-1+CD11b^{high}) (**A**) and activated T cells (CD3+CD4+CD44^{high}) (**B**), and flow cytometric analysis was performed as described in Materials and Methods. ** = $P < 0.025$; **** = $P < 0.0001$ by Student's unpaired *t*-test. **C** and **D**, To determine the effects of VISTA deficiency, 8-week-old B6, B6 *VISTA*^{-/-} (*VISTA*^{-/-}), *Sle1.Sle3*, and *Sle1.Sle3* *VISTA*^{-/-} (*Sle1,3*^{*VISTA*}) female mice (n = 6 per group) were monitored weekly for proteinuria (**C**) and survival (**D**). * = $P < 0.05$ versus *Sle1.Sle3* mice, by Student's unpaired *t*-test. **E**, Serum was collected at week 20 from the 4 groups of mice (n = 5 per group), and chemokine and cytokine expression was assessed as described in Materials and Methods. The heatmap was generated as described in Materials and Methods. Significant differences are indicated for *Sle1.Sle3* *VISTA*^{-/-} mice compared to B6, B6 *VISTA*^{-/-}, or *Sle1.Sle3* mice. Data are representative of 3 experiments. Values in **A**–**C** are the mean $\pm$ SEM. MCP-1 = monocyte chemoattractant protein 1; LIX = CXCL5; TNF = tumor necrosis factor; MIP-2 = macrophage inflammatory protein 2; IL-6 = interleukin-6; IFN γ = interferon- γ ; MIG = monokine induced by IFN γ ; IP-10 = IFN γ -inducible 10-kd protein.

Figure 2.

T cells from *Sle1.Sle3* VISTA^{-/-} mice are chronically activated. Splenocytes were harvested from 25-week-old B6, B6 VISTA^{-/-}, *Sle1.Sle3*, and *Sle1.Sle3* VISTA^{-/-} female mice (n = 6 per group). **A–D**, Cells were stained with directly conjugated antibodies to detect effector CD4⁺ T cells (**A**), activated CD4⁺ T cells (**B**), CD4⁺ Treg cells (**C**), and follicular helper T cells (**D**) and analyzed by flow cytometry. Symbols represent individual mice; bars show the mean ± SEM. * = P < 0.05; ** = P < 0.025 by Student's unpaired *t*-test. **E**, To investigate whether VISTA deficiency altered cytokine production, splenic CD3+CD4⁺ T cells harvested from *Sle1.Sle3* and *Sle1.Sle3* VISTA^{-/-} mice (n = 5 per group) were stimulated with CD3 and CD28 for 24 hours. Supernatants were harvested and screened as described in Materials and Methods. The heatmap was generated as described in Materials and Methods. Data are representative of 3 experiments. NS = not significant; PD-1 = programmed death 1 (see Figure 1 for other definitions).

Figure 3.

VISTA deficiency on the *Sle1.Sle3* background up-regulates costimulatory/activation molecules and proinflammatory cytokines in the splenic myeloid compartment. Splenocytes were harvested from 25-week-old B6, B6 VISTA^{-/-}, *Sle1.Sle3*, and *Sle1.Sle3* VISTA^{-/-} female mice (n = 5 per group). **A**, Cells were stained with directly conjugated antibodies to detect inflammatory monocytes. **B–D**, To determine the expression of costimulatory/activation molecules on lymphoid dendritic cells (DCs) (**B**), myeloid DCs (**C**), and macrophages (**D**), expression of CD80, CD40, and I-A^b was quantified by mean fluorescence intensity (MFI). **E–G**, To investigate cytokine production by F4/80+CD11b+Gr-1+ inflammatory myeloid cells, cells were stimulated for 24 hours with lipopolysaccharide (LPS), and supernatants were harvested and screened for the

proinflammatory cytokines IL-10 (**E**), IFN γ (**F**), and TNF (**G**) as described in Materials and Methods. Data are representative of 3 experiments. Symbols represent individual mice; bars show the mean $\pm$ SEM. * = $P < 0.05$; ** = $P < 0.025$; *** = $P < 0.005$; **** = $P < 0.0001$ by Student's unpaired t -test. NS = not significant (see Figure 1 for other definitions).

Figure 4.

VISTA deficiency exacerbates myeloid cell infiltration into the kidney. Female *Sle1.Sle3* and *Sle1.Sle3 VISTA^{-/-}* mice ages 25 weeks (n = 5 per group) were monitored weekly for proteinuria. **A**, To assess myeloid cell infiltration into the kidneys, immunofluorescence staining was performed on frozen kidney sections to detect CD11b+ and F4/80+ cells. Images were analyzed by confocal microscopy. Original magnification $\times 20$. **B**, To quantify myeloid cells by flow cytometry, renal cells were stained to identify Gr-1+ CD11b+ cells. Flow cytometric analysis was performed as described in Materials and Methods. Data are

representative of 3 experiments. Symbols represent individual mice; bars show the mean $\pm$ SEM. ** = $P < 0.025$ by Student's unpaired t -test. See Figure 1 for definitions.

Figure 5.

Heightened function of bone marrow–derived dendritic cells (BMDCs) and BM macrophages in *Sle1.Sle3* *VISTA*^{-/-} mice. **A** and **B**, To determine the activation status of *Sle1.Sle3* *VISTA*^{-/-} mouse cells characterized by CD80, CD16, I-A^b, and CD40, BMDCs (**A**) and BM macrophages (**B**) were harvested from B6, B6 *VISTA*^{-/-}, *Sle1.Sle3*, and *Sle1.Sle3* *VISTA*^{-/-} female mice (n = 6 per group) and stimulated for 24 hours with lipopolysaccharide (LPS). Cells were stained with directly conjugated antibodies to identify the molecules, and flow cytometric analysis was performed. **C–F**, In parallel, supernatants were harvested from LPS-activated BMDC cultures to screen for IL-12 (**C**) and TNF (**D**) and from LPS-activated BM macrophage cultures to assess levels of IL-12 (**E**) and TNF (**F**). Supernatants were screened as described in Materials and Methods. Data are representative of 3 experiments. Symbols represent individual mice; bars show the mean ± SEM. * = $P < 0.05$; ** = $P < 0.025$; *** = $P < 0.005$; **** = $P < 0.0001$ by Student's unpaired *t*-test. MFI = mean fluorescence intensity; NS = not significant (see Figure 1 for other definitions).

Figure 6.

VISTA deficiency exacerbates IFN signature in *Sle1.Sle3* mice. **A**, To assess levels of IFN α , serum was collected from B6, B6 VISTA $^{-/-}$, *Sle1.Sle3*, and *Sle1.Sle3* VISTA $^{-/-}$ female mice and tested with an IFN α enzyme-linked immunosorbent assay. **B–D**, To determine whether production of the IFN-associated cytokines CXCL10 and IFN β was increased in *Sle1.Sle3* VISTA $^{-/-}$ mice, bone marrow-derived dendritic cells isolated from the 4 groups of mice were stimulated with IFN α (**B**), R848 (which binds Toll-like receptor 7 [TLR-7]) (**C**), or CpG (which binds TLR-9) (**D**) for 24 hours. Supernatants were harvested and screened as described in Materials and Methods. Data are representative of 3 experiments. Symbols represent individual mice; bars show the mean $\pm$ SEM. * = $P < 0.05$; ** = $P < 0.025$; **** = $P < 0.0001$ by Student's unpaired *t*-test. NS = not significant (see Figure 1 for other definitions).